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(54) **Recombinant DNA**

(57) Recombinant DNA coding for the K99 antigen of enteropathogenic strains of *Escherichia coli*. Such recombinant DNA could be used to produce sufficient quantities of K99 antigen for incorporation in a vaccine against scouring, which is a diarrhoeal disease of adult and particularly neonatal animals. DNA coding for the *E. coli* K99 antigen can be obtained from the K99 plasmid of *E. coli* K12. The DNA can then be cloned in a high copy number plasmid such as pBR322 in *E. coli* C host cells and expressed to give the desired antigen.

GB 2 094 314 A

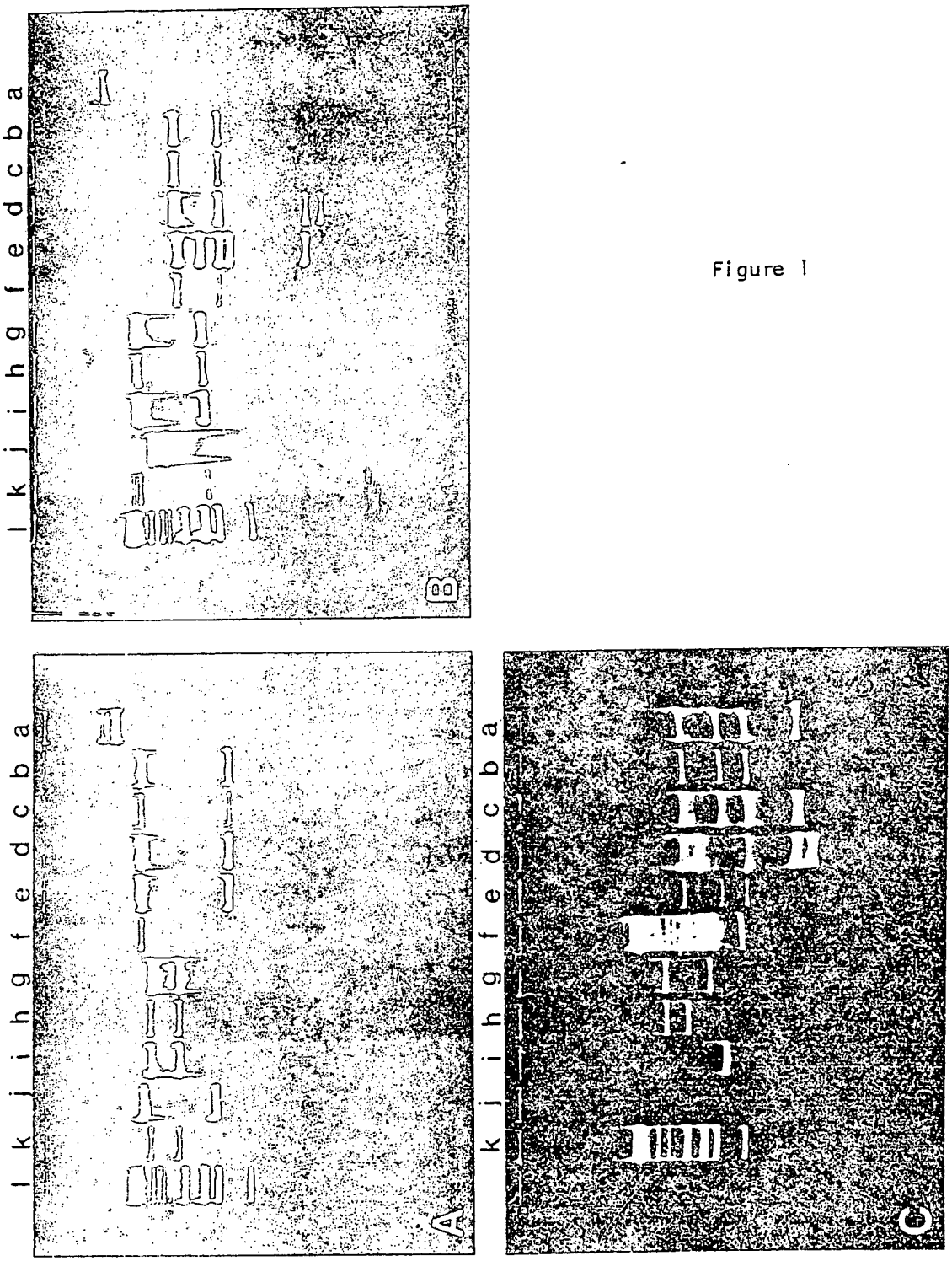


Figure 1

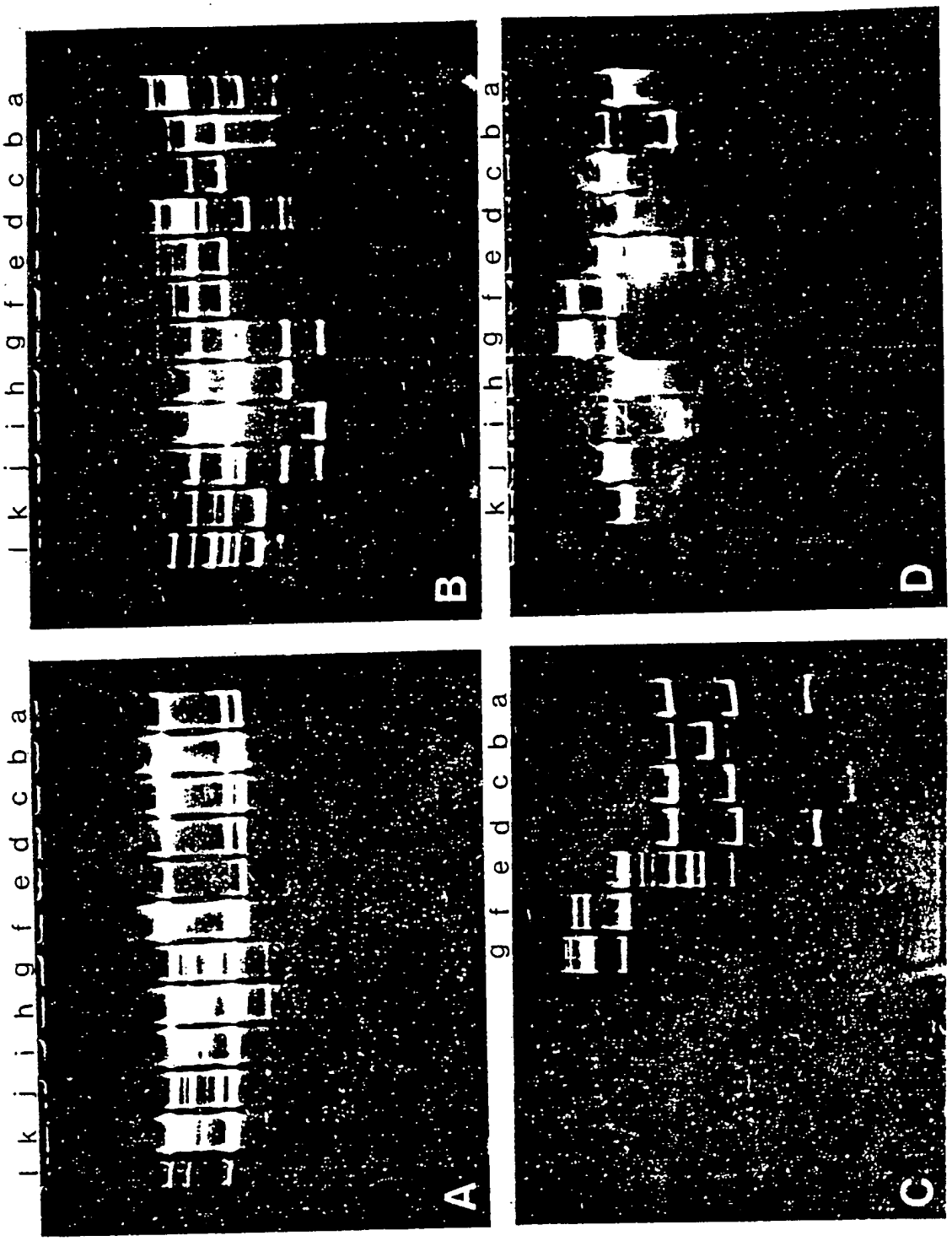


Figure 2

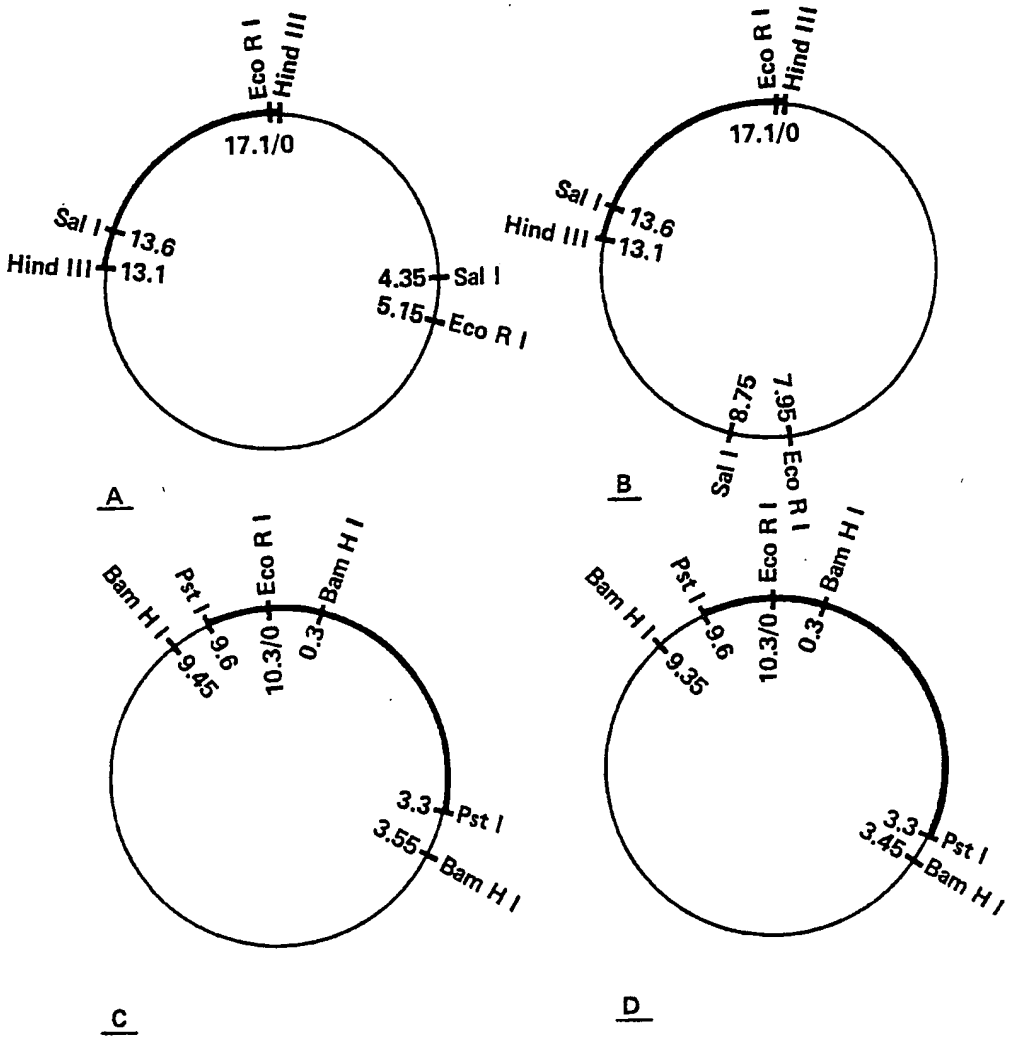
Eco R I
Hind III
17.1/0
Sal I
13.6
Hind III
13.1
4.35
Sal I
5.15
Eco R I
A
Eco R I
Hind III
17.1/0
Sal I
13.6
Hind III
13.1
8.75
7.95
Sal I
Eco R I
B
Bam H I
Pst I
Eco R I
Bam H I
9.6
9.45
10.3/0
0.3
3.3
Pst I
3.55
Bam H I
C
Bam H I
Pst I
Eco R I
Bam H I
9.6
9.35
10.3/0
0.3
3.3
Pst I
3.45
Bam H I
D

Figure 3

SPECIFICATION

# Recombinant DNA

The present invention relates to recombinant deoxyribonucleic acid (DNA) and, in particular, to recombinant DNA coding for an antigen of the bacterium *Escherichia coli*.

Scouring is a diarrhoeal disease in neonatal animals often caused by toxins released from enteropathogenic strains of *E. coli*, which can colonise the gut before the natural flora is established. Even adult animals, however, are susceptible to this form of diarrhoea when the natural balance of flora in the gut is upset, for example following antibiotic treatment or a sudden change in diet.

An important early stage in the pathogenesis of enterotoxigenic *E. coli* is the adhesion of the bacterium to the epithelium of the intestinal tract of the animal. Adhesion is mediated by specific capsular antigens present on the surface of the bacterial cell. Examples of such antigens, which have a polypeptide structure, include those designated as K88 and K99 antigens. The K88 antigen is primarily responsible for the adhesion of enteropathogenic strains of *E. coli* to the intestinal villi of piglets while the K99 antigen is primarily responsible for adhesion of enteropathogenic strains of *E. coli* to the intestinal villi of calves and lambs, and, to a lesser extent, of piglets. There are also substantial morphological differences between the K88 and K99 antigens. The K88 antigen is a fibrous capsule surrounding the cell, but the K99 antigen is a filamentous pilus. K99 and the various serotype of K88 also have important differing serological properties.

Using the techniques developed over the last five years it is now possible to introduce the DNA coding for specific proteins into bacterial cells via the intermediary of a plasmid or other cloning vehicle; see for example Burrell, C. J., *et al, Nature, 279,* 43—47, 1979. In general the construction of the recombinant DNA molecules comprises the steps of excising the DNA template coding for the desired protein from a parent cell and inserting this piece of heterologous DNA into a cloning vehicle, such as a bacterial plasmid, and then transforming an appropriate bacterial host with the modified plasmid. A general discussion of the manipulation of genes leading to the formation of recombinant DNA by S. Cohen was published in *Scientific American, 233,* 24—33, 1975.

Using the above techniques, study of the expression of the genetic determinant that encodes for one particular type of K88 antigen, namely K88ab, has been made by Mooi *et al* (Nucleic Acids Research *6*(3) 1979 (849)).

We have now discovered that genetic manipulation techniques may be applied to produce pilus-forming antigens of enteropathogenic strains of *E. coli*. The present invention is particularly but not exclusively concerned with the production of the K99 antigen which, for the reasons outlined above, forms an important immunological component of any vaccine for the treatment or prevention of *E. coli*-induced diarrhoeal disorders in calves, lambs and to some extent piglets.

Filamentous antigens of especially the K99 antigen of *E. coli* are generally associated with large, low copy number using conjugative plasmids. However, by using genetic manipulation techniques we have been able to incorporate the DNA nucleotide sequence coding for such antigens into smaller high copy number plasmids whereby increased levels of the antigen may be expressed upon replication.

For a better understanding of the present invention the following glossary is provided for the meaning of certain terms as used in the context of the present invention:—

Recombinant DNA:—
a hybrid double stranded DNA molecule comprising at least two double stranded DNA nucleotide sequences, one of the double stranded DNA nucleotide sequences not being found together in nature with the other, or at least one of the other, DNA nucleotide sequences.

Cloning vehicle:—
non-chromosomal double stranded DNA capable of replicating when placed in a unicellular microorganism.

Expression:—
the process involved in producing a polypeptide from a gene, e.g. a structural gene.

Plasmid:—
a cloning vehicle derived from viruses or bacteria.

Expression control sequence:—
a sequence of DNA nucleotides that controls or regulates the expression of a gene, e.g. a structural gene.

Structural gene:—
a sequence of DNA nucleotides which codes for a sequence of amino acids characteristic of a specific polypeptide.

According to a first aspect of the present invention, there is provided a recombinant DNA molecule comprising a nucleotide sequence substantially coding for a filamentous pilus antigen of an enteropathogenic strain of *Escherichia coli*.

The filamentous pilus antigen is desirably K99, and, therefore, there is further provided a recombinant DNA molecule comprising a nucleotide sequence substantially coding for the K99 antigen of an enteropathogenic strain of *E. coli*. Such a DNA molecule is capable of being expressed as a polypeptide recognisable as substantially corresponding to the K99 antigen.

The recombinant DNA molecule may also contain an expression control sequence or sequences preferably positioned adjacent to the nucleotide sequence coding for the pilus antigen. The control sequence(s) may be derived from *E. coli* DNA or from a heterologous source.

In the recombinant DNA molecules defined above, the nucleotide sequence substantially coding for the said filamentous pilus antigen is preferably present in a cloning vehicle, conveniently a plasmid having a relatively high copy number and/or a relatively low molecular weight compared with the parent plasmid from which the said sequence is derived. The sequence may be enzymatically excised or chemically synthesised. A preferred cloning vehicle is the plasmid pBR322.

According to a third aspect of the present invention, there is provided a host cell containing a recombinant DNA molecule as defined above.

According to a forth aspect of the present invention, there is provided a method of preparing recombinant DNA molecules as defined above, which comprises inserting a nucleotide sequence substantially coding for a filamentous pilus antigen of an enteropathogenic strain of *E. coli*, into a cloning vehicle.

In the above-described method the nucleotide sequence for the filamentous pilus antigen is preferably obtained by digestion with a restriction enzyme of a plasmid containing the desired sequence, e.g. a high molecular weight low copy number plasmid such as p TM1 obtained for example from *E. coli* K12. It has been found that *Hind* III restriction enzyme provides a fragment, of about 13.1 Kb (thousands of base pairs), which comprises a nucleotide sequence substantially coding for the desired antigen. *Hind* III cleaves the DNA of the original plasmid at sites where complementary nucleotides are arranged in rotational symmetry, the resulting fragments having what are known in the art as "cohesive" or "sticky" ends. The above-described fragments obtained by restriction enzyme digestion and containing the desired sequence of nucleotides substantially coding for a filamentous pilus antigen represent a further feature of the present invention.

The above-mentioned fragments comprising the desired nucleotide sequence can then be inserted in the cloning vehicle, e.g. a low molecular weight high copy number plasmid such as pBR322 which has previously been treated with a restriction enzyme to provide open ends between which the fragment containing the desired nucleotide sequence may be inserted and ligated, e.g. using a DNA ligase enzyme such as T4 ligase. Opening of the cloning vehicle is preferably effected using *Hind* III restriction enzyme and subsequent treatment with a phosphatase enzyme to remove terminal phosphate groups and thus prevent re-ligation. Other plasmids and indeed bacteriophages may be used as cloning vehicles, but pBR322 is preformed because of its ready availability and the relative ease with which it can be manipulated.

It will be seen from the Example given below that by using the genetic manipulation techniques outlined above, it is possible to obtain 5 different hybrid plasmid forms of the recombinant DNA according to the present invention. These 5 hybrid plasmids are identified by the codes pTP13, pTP23, pTP33, pTP43 and pTP53 and may be differentiated, as discussed below, on the basis of their differing behaviour on electrophoresis and enzyme digestion. Of the 5 hybrid plasmids, pTP13 and pTP43 are particularly preferred on account of their high copy number in comparison with pTM1, the naturally occuring K99 plasmid, and also their high level of K99 antigen expression in a host cell. The restriction enzyme maps for these two hybrid plasmids as shown in Figure 3 of the accompanying drawings which are discussed more fully below. These two hybrid plasmids, substantially as herein described, thus represent a preferred embodiment of the recombinant DNA according to the present invention.

After synthesis of the recombinant DNA molecules as defined above, an appropriate host, e.g. a bacterium such as *E. coli*, e.g. *E. coli* C, is transformed with the recombinant DNA which is then replicated in the host whereby the said nucleotide sequence for the filamentous pilus antigen is expressed as that antigen. A suitable strain of *E. coli* C is available from Dr. Barbara Bachman, Department of Microbiology School of Medicine, Yale University, New Haven, Connecticut, 06510, U.S.A.

The filamentous pilus antigen expressed by the recombinant DNA according to the present invention may be incorporated in a suitable vaccine for the prevention of *E. coli*-induced diarrhoeal disorders in lambs, calves and piglets. The formulation of such a vaccine may be carried out using techniques which are conventional in the vaccine art.

For a better understanding of the present invention, and to show how it may be put into effect, the following Example will now be given. Before the results of the Example are given, however, an explanation of the materials and methods used is given.

**Example**

**Bacterial strains**

A K99 positive exconjugant of *E. coli* K12 was used as a source of K99 plasmid. This plasmid was designated pTM1. The strain used for cloning the hybrid plasmids was *E. coli* C, it should be noted that *E. coli* B41 could also be used.

**Culture conditions**

In order to compare *E. coli* C strains containing different K99 hybrid plasmids, strains were grown in 20 ml of Trypticase Soy Broth (BBL Microbiology Systems, Cockeysville, Maryland, U.S.A.) in 100 ml baffled conical flasks which were shaken for 18—20 hr. at 140 cycles per min and 37°C. Strains were maintained on sheep blood/nutrient agar plates to which antibiotics had been added where appropriate.

**Isolation of plasmid DNA**

Plasmids were routinely prepared by growing bacteria in L-broth (tryptone, 1%; yeast extract, 0.5%; NaCl, 0.5%) overnight at 37°C. When pBR322 and hybrid derivatives of pBR322 were prepared, chloramphenicol (200 ug/ml) was added to the bacterial cultures when they had reached an optical density (OD)650 of 0.1 and then incubated for a further 18 hr. The bacteria were then centrifuged (7 700 g, 5 min), resuspended in 3.0 ml of 25% (w/v) sucrose in 0.05 M Tris buffer (pH 8.0) and 0.5 ml of lysozyme (5 mg/ml in 0.25 M Tris buffer, pH 8.0) and 1.0 ml of ethylenediaminetetraacetic acid (EDTA) (0.25 M, pH 8.0) were added. After 5 min, 4.0 ml of Brij 58 lysis mixture was added (Brij 58, 1%; sodium deoxycholate, 0.4%; EDTA, 0.0625 M; Tris buffer, 0.05 M (pH 8.0)). After 3—10 incubation at 4°C, the lysed bacteria were centrifuged (48 000 g, 15 min).

Equilibrium centrifugation in CsCl/ethidium bromide was then carried out by mixing 7.0 ml of the supernatant with 7 g of CsCl and 0.875 ml of ethidium bromide (5 mg/ml). The mixture was transferred to a 10 ml centrifuge tube, overlayed with mineral oil and centrifuged at 40 000 rpm for at least 40 hr a 19°C. After centrifugation, plasmid bands were located by means of a UV (366 nm) light and removed with a syringe and needle. Ethidium bromide was subsequently removed from the plasmid preparations by two extractions with iso-propanol saturated with CsCl and the latter was then removed by dialysis against several changes of Tris buffer (10 mM, pH 7.6).

**Enzymes and chemicals**

Restriction enzymes, T4 DNA ligase and bacterial alkaline phosphatase as well as agarose were purchased from Bethesda Research Laboratories. ATP, dithiothreitol, ethidium bromide, Tris base, Brij 58, lysozyme and RNase were purchased from Sigma.

**Cleavage of DNA and cloning procedures**

For characterisation, plasmid DNA was cleaved by restriction enzymes in conventional manner. For the purpose of cloning, DNA was digested for 1—2 hr at 37°C in 66.0 mM Tris buffer pH 7.7 which was made 10.0 mM with respect to $MgCl_2$. The vector plasmid, pBR322 was treated with alkaline phosphatase at the same time as restriction enzyme digestion to remove terminal phosphate groups. Enzymes were then inactivated by incubation at 65°C for 10 min. Having checked that the vector was unable to re-ligate to the open circle form, cloning of the restriction enzyme fragments of the K99 plasmid from *E. coli* K12 was carried out by adding one fifth the quantity of pBR322 digested with the same restriction enzyme as well as alkaline phosphatase, followed by ATP to a final level of 0.4 mM, dithiothreitol to a final level of 10.0 mM and 1—2 ul of T4 DNA ligase. After overnight incubation at room temperature (20°C), the DNA ligase was inactived by incubation at 65°C for 10 min.

**Transformation procedure**

For transformation, a 25.0 ml shake flask culture of *E. coli* C was grown in nutrient broth at 37°C and 140 cycles/min until the OD590 was 0.5—0.6. At this stage, the culture was chilled in ice water, centrifuged (8 000 rpm for 5 min at 4°C), washed with 25.0 ml of chilled $CaCl_2$ (0.03 M) and resuspended in 25.0 ml of chilled $CaCl_2$. After 20 min incubation on ice, the cells were centrifuged and resuspended in 2.0—5.0 ml of chilled $CaCl_2$. The 20—50 ul of DNA (containing 1—1 000 ng) was added to 0.2 ml of the chilled cell suspension followed by 0.1 ml of $CaCl_2$. After 30 min incubation on ice the bacteria were 'heat pulsed' by 3 min incubation at 42°C with agitation. Then 3.0 ml of L-broth (made 0.2% with respect to glucose) was added and the cells were incubated for 90 min at 37°C before being plated on appropriate selective media at dilutions ranging from 0—$10^{-2}$. Cell controls to which DNA had not been added were routinely run at the same time to ensure that the recipient strain of *E. coli* remained sensitive to the selective media in the absence of transformation.

**Agarose gel electrophoresis**

Agarose slab gels (13x14x0.5 cm) 0.8% were made up in electrode buffer (Tris base, 1.08%; NaEDTA, 0.093% and boric acid 0.55% (pH 8.0)). Gels were either run for 3 hr at 60 mA and approximately 80 V or overnight at 12 mA and 20 V, stained in ethidium bromide (1.0 ug/ml) for 30

min and then examined on a Chromoto-Vue transilluminator (model C62, Ultra Violet Products Inc.). Gels were photographed using Kodak Pan Lithographic film and Wratten (No 15) and orange filters.

To detect smaller DNA fragments, 1.4% agarose gels made up in Tris/acetate buffer (20.0 mM, pH 7.7) containing 2.0 mM EDTA. The gels were run at 60 mA for 3—3.5 hr after which time the bromophenol blue indicator dye had moved 75% the length of the gel.

**K99 detection and assay**

Colonies which were positive for K99 antigen were normally detected by slide agglutination.

K99 antigen was assayed by a back titration procedure in which bacterial cultures were serially diluted in 0.5 ml of formol (0.25%) saline in small glass tube (8×0.7 (diam) cm). Half a millilitre of a standard anti-K99 antiserum was then added to each tube and, after thorough mixing, the tubes were incubated for 60 min at 37°C. The tubes were then centrifuged, 50 ul of the supernatant from each tube were placed in U-shaped wells in a plastic tray and 50 ul of a standard suspension of K99 positive *E. coli* were finally added to each well. The plastic trays were then wrapped in a plastic bag to prevent dehydration and incubated at 37°C overnight. Where the bacterial suspension under test had adsorbed all the anti-K99 antiserum, the standard suspension of K99 positive *E. coli* formed a compact 'button' on the bottom of the well. Conversely, in the absence of complete absorption the remaining anti-K99 antibody agglutinated the test suspension and prevented 'button' formation.

**Copy number determination**

To determine plasmid copy number, *E. coli* C transformants were grown in 20.0 ml of M9 medium ($Na_2HPO_4$, 0.6%; $KH_2PO_4$, 0.3%; NaCl, 0.05%; $NH_4Cl$, 0.1% and $CaCl_2$ added to 0.1 mM) containing 0.2% glucose and 0.5% casamino acids (Difco) at 37°C and 140 cycles per min. When the cell concentration had reached OD650 of 0.1, methyl tritiated thymidine was added to a final concentration of 1.0 uCi/ml as well as unlabelled thymidine to 2.0 ug/ml and deoxyadenosine to 200 ug/ml (Boyce and Setlow, *Biochim. Biophys. Acta 61*, 618—620, (1962)). The cultures were then incubated overnight at 37°C, harvested by centrifugation (10 000 g, 10 min at 4°C) and washed twice with 20.0 ml volumes of TES buffer (Tris, 50 mM; NaCl, 50 mM; EDTA 5 mM; pH 8.0). Cells pellets were then resuspended in 0.1 ml of 20.0% sucrose to which 40 ul lysozyme (5 mg/ml) and 60 ul of RNase (1 mg/ml) were added. The cells were incubated at 37°C for 30 min after which cell lysis was accomplished by adding 200 ul of Brij lysis mixture, incubating for a further 30 min at 37°C and then shearing the cells through a 25 gauge needle.

The lysates were made up to 8.0 ml with TES buffer and then 8.0 g of CsCl and 0.2 ml of ethidium bromide (10 mg/ml) were added. The lysates were centrifuged at 40 000 rpm for a minimum of 40 hr at 19°C, after which holes were punched in the bottom of the centrifuge tubes and fractions (0.3 ml) were collected. Ten microlitre aliquots of each fraction were placed on filter paper squares (1.5×1.5 cm) which were dried, treated twice with cold TCA (5%), washed with ethanol and dried again. Each filter paper square was placed in a scintillation vial, 5.0 ml of scintillation fluid (Koch Light, KL502) was added and the vials counted on a Beckman scintillation counter.

**Results**

The results obtained are illustrated by reference to the accompanying drawings, in which:—

Figure 1 shows agarose gels (0.8%) illustrating the result of the following restriction enzyme digests of the hybrid plasmids pTP13—53:

*A* (a) undigested pTP13; (b) *Hind* III digest of pTP13; (c) *Hind* III digest of pTP23; (d) *Hind* III digest of pTP33; (e) *Hind* III digest of pTP43; (f) *Hind* III digest of pTP53; (g) *Sal* 1 digest of pTP13; (h) *Sal* 1 digest of pTP23; (i) *Sal* 1 digest of pTP33; (j) *Sal* 1 digest of pTP43; (k) *Sal* 1 digest of pTP53; (l) *Eco* R1 digest of —DNA;

*B* (a) undigested pTP13; (b) *Bam* H1 digest of pTP13; (c) *Bam* H1 digest of pTP23; (d) *Bam* H1 digest of pTP33; (e) *Bam* H1 digest of pTP43; (f) *Bam* H1 digest of pTP53; (g) *Eco* R1 digest of pTP13; (h) *Eco* R1 digest of pTP23; (i) *Eco* R1 digest of pTP33; (j) *Eco* R1 digest of pTP43; (k) *Eco* R1 digest of pTP53; (l) *Eco* R1 digest of —DNA;

*C* (a) *Pst* 1 digest of pTP13; (b) *Pst* 1 digest of pTP23; (c) *Pst* 1 digest of pTP33; (d) *Pst* 1 digest of pTP43; (e) *Pst* 1 digest of pTP53; (f) *Eco* R1 digest of —DNA; (g) *Bam* H1 digest of pTP11; (h) *Bam* H1 of pTP12; (i) *Pst* 1 digest of pBR322; and (k) *Eco* R1 digest of —DNA;

Figure 2 shows agarose gels (0.8%) illustrating the results of the following restriction enzyme digests of those plasmids derived from pTP13 and pTP43 either by double digestion followed by religation or subcloning a *Pst* 1 fragment of pTP13 and pTP43:

*A* (a) *Hind* III/*Sal* 1 digests of pTP13; (b) *Hind* III/*Sal* 1 digest of pTP131S; (c) *Hind* III/*Sal* 1 digest of pTP132S; (d) *Hind* III/*Sal* 1 digest of pTP43; (e) *Hind* III/*Sal* 1 digest of pTP431S; (f) *Hind* III/*Sal* 1 digest of pTP432S; (g) *Eco* R1/*Hind* III digest of pTP13; (h) *Eco* R1/*Hind* III digest of pTP131E; (i) *Eco* R1/*Hind* III digest of pTP132E; (j) *Eco* R1/*Hind* III digest of pTP43; (k) *Eco* R1/*Hind* III digest of pTP431E; (l) *Eco* R1/*Hind* III digest of pTP432E;

*B* (a) *Pst* 1 digest of pTP13; (b) *Pst* 1 digest of pTP131; (c) *Pst* 1 digest of pTP132; (d) *Pst* 1 digest of pTP43; (e) *Pst* 1 digest of pTP432; (f) *Pst* 1 digest of pTP433; (g) *Bam* H1/*Hind* III digest of

pTP13; (h) *Bam* H1/*Hind* III digest of pTP131B; (I) *Bam* H1/*Hind* III digest of pTP132B; (j) *Bam* H1/*Hind* III digest of pTP43; (k) *Bam* H1/*Hind* III digest of pTP431B; (l) *Bam* H1/*Hind* III digest of pTP432B;

*C* (a) *Bam* H1 digest of pTP131; (b) *Bam* H1 digest of pTP132; (c) *Bam* H1 digest of pTP432; (d) *Bam* H1 digest of pTP433; (e) *Eco* R1 digest of —DNA; (f) undigested pTP131; (g) undigested pTP432.

*D* (a) *Sal* 1 digest of pTP131; (b) *Sal* 1 digest of pTP132; (c) *Sal* 1 digest of pTP432; (d) *Sal* 1 digest of pTP433; (e) *Eco* R1 digest of —DNA; (f) undigested pTP131; (g) undigested pTP432; (h) *Eco* R1 digest of pTP131; (i) *Eco* R1 digest of pTP132; (j) *Eco* R1 digest of pTP432; and (k) *Eco* R1 digest of pTP433; and

Figure 3 shows restriction enzyme maps of the hybrid plasmids which code for the K99 antigen. The heavy lines represent pBR322 and the figures indicate the size of the fragments in kilobases. A is pTP13, B is pTP43, C is pTP432, D is pTP131 and pTP433.

**Cloning of DNA coding for the K99 antigen**

Plasmid DNA prepared from *E. coli* K12 which contained a large, conjugative plasmid coding for the K99 antigen (designated pTM1) was digested with *Hind* III restriction enzyme. The vector plasmid pBR322 was also digested with *Hind* III as well as alkaline phosphatase to remove terminal phosphate groups to prevent religation without fragment incorporation.

Vector plasmid and the *Hind* III digest were ligated after ATP, dithiothreitol and T4 DNA ligase addition. Competent cells of *E. coli* C were then transformed with this recombinant DNA preparation and amplicillin resistant transformants were screened for K99 formation by slide agglutination. Plasmid DNA was prepared from five transformants which were K99 positive and tetracycline sensitive in order to characterise the DNA fragments which had been cloned into pBR322. The five hybrid plasmids were designated pTP13—pTP53.

The results shown in Figure 1A indicate that all five DNA preparations yielded *inter alia* the same two fragments on *Hind* III digestion. One of these was linear pBR322 and the other, a 13.1 kb fragment of pTM1. Digestion of the hybrid plasmids with *Sal* 1, *Eco* R1, *Bam* H1 and *Pst* 1 all indicated that the orientation of the cloned fragments were identical except for pTP43 (Figure 1B and 1C). As the expression of K99 in *E. coli* C (pTP43) appeared, on the basis of slide agglutination, to be as good as expression in any of the other *E. coli* C strains containing the other hybrid plasmids, there was a clear indication that the expression of K99 was independent of the orientation of the K99 genetic determinants with respect to the vector plasmid. The digestion data also indicated that the inserted fragments had only one *Eco* R1 and one *Sal* 1 site but at least five *Bam* H1 and five *Pst* 1 sites. These results are summarised in the restriction enzyme maps for pTP13 and pTP43 shown in Figures 3A and 3B respectively.

**Sub-cloning of the K99 genetic determinants**

As the *Hind* III fragment was large (13.1 kb) in comparison to the vector plasmid and evidently contained sites for *Bam* H1, *Sal* 1, *Eco* R1 and *Pst* 1, it was decided to attempt isolation of a smaller fragment in the hope that the resulting hybrid plasmid would have a higher copy number and, therefore, possibly give a higher level of K99 expression. The plasmids pTP13 and pTP43 were simultaneously digested with *Hind* III and either *Sal* 1, *Bam* H1, or *Eco* R1. After inactivation of the restriction enzymes the resulting digests were re-ligated and the DNA preparations then used to transform competent cells of *E. coli* C. As before, ampicillin resistant transformants were tested for K99 formation by slide agglutination. In all cases it was possible to isolate K99 positive transformants. Plasmids derived from *Hind* III/*Eco* R1 digest of pTP13 were designated pTP131E, pTP132E etc and those derived from pTP43 were designated pTP431E, pTP432E etc. Similarly plasmids derived from *Hind* III/*Bam* H1 digests were designated pTP131B, pTP132B, pTP431B and pTP432B and those derived from *Hind* III/*Sal* 1 digests were designated pTP131S, pTP132S, pTP431S and pTP432S. Restriction enzyme digests of plasmid DNA prepared from these strains revealed that, in some cases, the pattern of bands were identical to that of the parent hybrid plasmid, but, in other cases, certain fragments had been eliminated (Figure 2A). Only those hybrid plasmids which were known to be smaller than the parent hybrid plasmids (pTP13 and pTP43) were retained for further studies on copy number estimation and K99 expression.

Because the *Pst* 1 site in pBR322 occurs in the ampicillin resistance gene (unlike the *Bam* H1, *Sal* 1 and *Hind* III sites which all occur in the tetracycline resistance gene) it was not possible to adopt the strategy of double digestion with *Pst* 1 and *Hind* III as this would have destroyed resistance to both tetracycline and ampicillin. Consequently, *Pst* 1 digests of both pTP13 and pTP43 were mixed with pBR322 which had been digested with *Pst* 1 and treated with alkaline phosphatase and the resulting mixture was ligated. Competent cells of *E. coli* C were transformed with this DNA preparation and tetracycline resistant colonies were screened for K99 production. Four K99 positive transformants were found; two (designated pTP131 and pTP132) contained hybrid plasmids with *Pst* 1 fragments from pTP13 and the other two (designated pTP432 and pTP433) contained *Pst* 1 fragments from pTP43. Plasmid DNA was prepared from the *E. coli* C strains containing pTP131, 132, 432 and 433 and digested with *Bam* H1, *Eco* R1 and *Sal* 1 (Figure 2B) as well as *Pst* 1. The *Pst* 1 digests revealed that all four hybrid plasmids contained one 6.3 Kb fragment inserted into the *Pst* 1 site of pBR322. The

hybrid plasmids contained one *Eco* R1 and one *Sal* 1 site as well as three *Bam* H1 sites. As pBR322 is known to contain one *Eco* R1, one *Sal* 1 and one *Bam* H1 site, it was concluded that the inserted fragment had no *Eco* R1 or *Sal* 1 site but two *Bam* H1 sites. From the *Bam* H1 digest (Figure 2) it was also apparent that the orientation of the fragment was identical in pTP131 and pTP433 but opposite in pTP432. The restriction enzyme maps for pTP131, pTP432 and pTP433 are shown in Figures 3C and 3D of the accompanying drawings. The results obtained with pTP132 could not be readily interpreted and after several subcultures the strain containing this plasmid failed to produce K99.

**Copy number determinants of hybrid plasmids**

Copy number estimations were carried out on pBR322, pTP13, pTP43, pTP131, pTP132, pTP432 and pTP433 as described in the earlier section headed "Copy Number Determination". The results given in the Table below show that the copy number of pTP13 and pTP43 were roughly one third that of pBR322. Estimations of pTP132 and pTP433 repeatedly indicated that the number of copies was similar to those of pTP13 and pTP43, but the number of copies of pTP131 and pTP432 were significantly higher.

**The expression of the K99 pilus antigen**

In order to compare the level of K99 antigen expressed by the original plasmid isolated from *E. coli* K12 (pTM1) with the levels expressed by the hybrid plasmids containing the K99 genetic determinants, it was necessary to transfer the plasmid pTM1 into *E. coli* C. This was of added importance because unlike *E. coli* K12 the K99 formed by *E. coli* C does not deteriorate in stationary phase when cultured in Trypticase Soy Broth (Isaacson, *Inf. and Immun. 28* 190—194 (1980)). Consequently, *E. coli* K12 (pTM1) was transformed with pBR322 and one of the resulting transformants was mated with a strain of *E. coli* C which was resistant to high levels of rifampicin. Exconjugants of *E. coli* C which had received both pTM1 and pBR322 (by co-migration) were selected by plating on agar containing both ampicillin (50 ug/ml) and rifampicin (100 ug/ml) and further characterised by their requirement for methionine when grown on a minimal medium.

The *E. coli* C strains containing the various hybrid plasmids as well as pTM1 were cultured in Tryticase Soy Broth and then assayed for the K99 antigen as described above. The results are given in the following Table.

**Table**

The production of K99 pilus antigen by strains of *E. coli* C containing K99 hybrid plasmids when grown in Trypticase Soy Broth to stationary phase.

| *Plasmid* | *OD650 overnight culture* | *K99 (unit/ml)* | *K99/OD650* | *Plasmid Copy No*[1] | *Mol. Wt (MD)* |
|---|---|---|---|---|---|
| pTM1+pBR322 | 3.7 | 11.4—17.1 | 3.1—4.6 | —[2] | — |
| pTP13 | 4.4 | 384.4—576.7 | 87.4—131.0 | 22 | 11.12 |
| pTP43 | 4.3 | 384.4—576.7 | 89.4—134.0 | 25 | 11.12 |
| pTP131 | 4.1 | 171.0—256.3 | 41.7—62.5 | 59 | 6.7 |
| pTP432 | 3.6 | 171.0—256.3 | 47.5—71.2 | 51 | 6.7 |
| pTP433 | 4.0 | 144.0—171.0 | 28.9—43.3 | 29 | 6.7 |
| pTP131E | 4.1 | 384.4—576.7 | 93.8—140.7 | — | — |
| pTP431E | 4.5 | 384.4—576.7 | 85.4—128.2 | — | — |
| pTP431S | 3.1 | 50.6—76.0 | 16.3—24.5 | — | — |
| pTP131B | 4.1 | 256.3—384.4 | 62.5—93.8 | — | — |
| pTP431B | 5.0 | 171.0—256.3 | 34.2—51.3 | — | — |

[1]Copies per chromosome—mean of two or three determinations.
[2]The average copy number estimation of pBR322 was 76.

It was found that the highest levels of K99 expression were given by *E. coli* C (pTP13) and *E. coli* C (pTP43) which produced almost thirty times as much K99 antigen as *E. coli* C (pTM1). *E. coli* C containing pTP131, 132, 432, or 433 failed to produce as much K99 antigen as either *E. coli* C (pTP13) or *E. coli* C (pTP43) even though the number of copies of pTP131 and pTP432 were significantly higher than pTP13 or pTP43.

Expression of the K99 antigen by the plasmids pTP131E and pTP431E which were made by ligation of pTP13 and 43 after digestion with both *Hind* III and *Eco* R1, gave results as high as pTP13. The other plasmids, pTP431S (formed by ligation of a *Sal* 1/*Hind* III digest of pTP43) and pTP131B and pTP431B (formed by ligation of *Bam* H1/*Hind* III digests of pTP13 and 43 respectively) failed to produce as much K99 antigen as pTP13 or pTP43. In this respect they appear to be similar to pTP131, pTP432 and pTP433.

**Discussion**

In this example, a 13.1 Kb fragment of the plasmid pTM1 which codes for the K99 antigen was cloned into the multicopy plasmid pBR322. Restriction enzyme analysis of the hybrid plasmids containing this fragment indicated that four or five (pTP13, pTP23, pTP33 and pTP53) were identical while the remaining plasmid (pTP43) had the DNA fragment in the opposite orientation with respect to the cloning vector. Examination of the levels of K99 antigen production by *E. coli* C strains containing pTP13 and pTP43 demonstrated that the expression of K99 was independent of orientation (Table). This result was in contrast to previous work on the K88ab genetic determinants where *Hind* III fragment of plasmid DNA isolated from all six K88ab positive clones were found to be in the same orientation. Mooi *et al* (*loc. cit*) had also found that K88ab expression from an 11.8 Kb *Hind* III fragment cloned into pBR322 was highly orientation dependent.

The expression of K99 by *E. coli* C (pTP13) or *E. coli* C (pTP43) was approximately thirty times the level produced by *E. coli* C (pTPM1). This correlates well with the copy numbers of pTP13 and pTP43 which were high in relation to pTM1 (the low copy, K99 plasmid from which the 13.1 Kb *Hind* III fragment was originally obtained) but only about a third of the copy number of pBR322.

**Claims**

1. A recombinant DNA molecule comprising a nucleotide sequence substantially coding for a filamentous pilus antigen of an enteropathogenic strain of *Escherichia coli*.

2. A recombinant DNA molecule comprising a nucleotide sequence substantially coding for the K99 antigen of an enteropathogenic strain of *E. coli*.

3. A recombinant DNA molecule according to claim 1 or 2, which molecule comprises one or more expression control sequences.

4. A recombinant DNA molecule according to claim 3, wherein the or at least one of the expression control sequences is positioned adjacent to the nucleotide sequence substantially coding for the antigen.

5. A recombinant DNA molecule according to claim 3 or 4, wherein the or at least one of the control sequences is derived from *E. coli* DNA.

6. A recombinant DNA molecule according to claim 3 or 4, wherein the or at least one of the control sequences is derived from a source that is heterologous to *E. coli*.

7. A recombinant DNA molecule according to any one of claims 1 to 6, wherein the nucleotide sequence substantially coding for the antigen is present in a cloning vehicle.

8. A recombinant DNA molecule according to claim 7, wherein the cloning vehicle is a plasmid having a relatively high copy number and/or a relatively low molecular weight compared with a parent plasmid from which the nucleotide sequence substantially coding for the antigen may be derived.

9. A recombinant DNA molecule according to claim 7 or 8, wherein the cloning vehicle is the plasmid pBR322.

10. A host cell containing a recombinant DNA molecule substantially coding for a filamentous pilus antigen of an enteropathogenic strain of *E. coli*.

11. A method of preparing a recombinant DNA molecule substantially coding for a filamentous pilus antigen of an enteropathogenic strain of *E. coli*, which method comprises inserting a nucleotide sequence substantially coding for a filamentous pilus antigen of an enteropathogenic strain of *E. coli* into a cloning vehicle.

12. A method according to claim 11, wherein the nucleotide sequence is obtained by digestion with a restriction enzyme of a plasmid containing the desired sequence.

13. A method according to claim 12, wherein the plasmid is the pTM1 plasmid obtainable from *E. coli* K12.

14. A method according to claim 11 or 12, wherein the restriction enzyme is *Hind* III.

15. A hybrid plasmid identified by the code pTP13, pTP23, pTP33, pTP43 or pTP53.

16. A hybrid plasmid identified by the code pTP13 or pTP43.

17. A proteinaceous expression product of a recombinant DNA molecule according to any one of claims 1 to 9, 15 and 16.

18. A vaccine comprising a proteinaceous expression product according to claim 17.

19. A recombinant DNA molecule comprising a nucleotide sequence substantially coding for a filamentous pilus antigen of an enteropathogenic strain of *E. coli* substantially as hereinbefore described with reference to the Example.

20. A host cell containing a recombinant DNA molecule substantially coding for a filamentous pilus antigen of an enteropathogenic strain of *E. coli* substantially as hereinbefor described with reference to the Example.

21. A method of preparing a recombinant DNA molecule substantially coding for a filamentous pilus antigen of an enteropathogenic strain of *E. coli* substantially as hereinbefore described with reference to the Example.

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